GPNMB: A Molecular Target For Human High-Grade Glioma Immunotherapy

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Abbreviations Used: HGL, high-grade glioma; AA, anaplastic astrocytoma; GBM, glioblastoma multiforme; MED, medulloblastoma; MAb, monoclonal antibody; GPNMB, glycoprotein nmb; RT-PCR, reverse-transcription polymerase chain reaction; FACS, fluorescence-activated cell sorting; QFACs, quantitative FACS.

ABSTRACT

Targeting neoplastic high-grade glioma (HGL) cells for treatment with monoclonal antibody (MAb) constructs requires surface markers expressed by glioma cells but sparing normal brain. We have identified promising molecular HGL tumor targets, human transmembrane glycoprotein *nmb* ($GPNMB_{wt}$) and a splice variant form ($GPNMB_{sv}$, a 12 amino acid in-frame insertion in the extracellular domain). Quantitative real-time PCR on 28 newly diagnosed HGL patient tumor samples indicated that 19 of 25 GBM (76%) and 2 of 3 AA (67%) were positive for $gpnmb_{wt+sv}$ transcripts and 7 of 25 GBM (28%) and 2/3 of AA (67%) were positive for $gpnmb_{sv}$ transcripts. Normal brain samples expressed little or no *gpnmb* mRNA. We have obtained and characterized an anti-$GPNMB_{sv}$ polyclonal rabbit antiserum (#2640) and three MAbs (A3 [IgG_1] and G11 and U2 [IgG_{2b}]). IHC analysis with antiserum #2640 and MAb G11 detected GPNMB in a membranous and cytoplasmic pattern in 39 of 60 GBM (65%) and 12 of 17 AA (71%), with focal perivascular reactivity in approximately 30% of GBM cases. The binding affinity constants of the MAbs ranged from 2.7 - 96 $\times$ 10^7 M^{-1} (BIAcore vs. GPNMB), or 1.7 - 4.7 $\times$ 10^8 M^{-1} (Scatchard analyses vs. cell expressed GPNMB). Quantitative flow cytometric analysis of 12 GPNMB-positive HGL biopsy specimens (11 GBM, 1 AA) revealed GPNMB cell surface molecular density of 1.1-7.8 $\times$ 10^4 molecules/cell, levels sufficient for MAb targeting. Univariate and multivariate analyses correlated expression of GPNMB with survival utilizing IHC data (77 HGL patients) and RNA expression data (28 HGL patients), establishing that patients with relative high/moderate mRNA *gpnmb* transcript levels greater than ~2- to 3-fold over normal brain also have a higher risk of death (hazard ratio of 5 to 7). Increased *gpnmb* mRNA levels also correlated with elevated GPNMB protein expression in HGL biopsy samples. High *gpnmb* RNA levels are prognostic of poorer survival, and low levels prognostic of

significantly better survival. These data indicate that GPNMB is a potentially useful tumor-associated antigen and prognostic predictor for therapeutic approaches with malignant gliomas.

Introduction

High-grade gliomas (HGLs), including anaplastic astrocytoma (AA) and glioblastoma multiforme (GBM), account for about 50% of all primary brain tumors in adults (1). Despite recent advances in surgery and radiotherapy and the development of chemotherapeutic reagents, the clinical outcome for most HGL patients is unsatisfactory. The 2-year survival rate for GBM, the most malignant form, is less than 15-20 %, which has remained substantially unchanged over the last two decades (2).

Monoclonal antibody (MAb)-based targeted therapy has been introduced to circumvent the limited efficacy of conventional therapies (3). Either alone or in conjunction with cytotoxins or radioisotopes, antibodies that are developed against cell surface or extracellular matrix antigens with tumor-restricted distribution can offer a more specific delivery system for cytotoxic reagents resulting in a high degree of selective destruction of tumors. The search for appropriate brain tumor-associated antigens has been a key challenge for immunotherapeutic approaches to central nervous system (CNS) neoplasia (4-8). Development and progression of glial tumors arises as the result of accumulation of multiple genetic alterations in a single cell. As a result, malignant gliomas are composed of heterogeneous populations of cells both in genotype and phenotype (9, 10). Therefore, glioma cells exhibit a wide variation in antigenic profile even within each individual tumor (11). No single gene product has been reported to be overexpressed in all HGL cases. Success in MAb-based therapy for HGL will require the identification of a panel of glioma-associated antigens and the proper selection of target-specific MAbs for each patient.

Recent advances in the development of comprehensive molecular analysis tools for genome and gene expression provide a basis to discover novel target molecules with tumor-

specific distribution (12). In an attempt to identify novel glioma-associated antigens, we have previously reported several genes that are preferentially expressed in HGLs by the serial analysis of gene expression method (13). Among these candidate HGL marker genes, *glycoprotein nmb* (*GPNMB*) showed a greater than 10-fold induction of mRNA expression over normal brain samples in 5/12 of HGL cases (13).

GPNMB is a type I transmembrane protein which was isolated from a subtractive cDNA library based on differential expression between human melanoma cell lines with low and high metastatic potential in nude mice. *gpnmb* mRNA was expressed at high levels in low-metastatic melanoma cell lines and xenografts (14). The human *gpnmb* gene encodes a predicted 560-amino acid protein, the deduced amino acid sequence of which shows that GPNMB is made up of three domains, a long extracellular domain (ECD) preceded by a signal peptide, a single transmembrane region, and a relatively short cytoplasmic domain (Fig. 1A). The human GPNMB amino acid sequence had homology of 71.1% to DC-HIL (15), 69.8% to Osteoactivin (16), 56% to the precursor of pMel 17 (17), and 51% to QNR-71 (18). The human GPNMB gene was localized to human chromosome 7q15 (NCBI Unigene Cluster Hs.82226 *GPNMB*), a locus involved in the human inherited disease cystoid macular dystrophy. Recently, Bachner et al suggested that human *GPNMB* may be a candidate gene for the dominant cystoid macular edema since they found high expression of murine *gpnmb* mRNA within the retinal and iris pigment epithelium (19). The function of GPNMB has not been fully described, and paradoxical effects have been noted in transfection studies. Transfection of our *in vitro* minimally transformed human fetal astrocyte line THRG (20) with *gpnmb* cDNA altered the phenotype of both subcutaneous and intracranial tumors growing in athymic mice from a minimally invasive to a highly invasive and metastatic phenotype. Conversely, transfection of a partial *gpnmb*

cDNA into a highly metastatic melanoma cell line resulted in slower subcutaneous tumor growth and also in reduction of the potential for spontaneous metastasis in nude mice (14).

If the overexpression of *GPNMB* RNA by gliomas and lack of expression in normal brain is reflective of *GPNMB* protein expression, *GPNMB,* as an integral membrane protein, could be an important target for immunotherapy. Moreover, there is evidence that *GPNMB* may be involved in the invasive malignant phenotype of gliomas, making evaluation of *GPNMB*-expressing cells important (21). In our previous analysis, we could not detect *gpnmb* transcripts in four normal brain cortex samples, two whole brain samples, or one sample each of cerebellum, spinal cord, heart, kidney, lung, trachea, tonsil, or bone marrow (13). We have therefore proposed to investigate the suitability of this *gpnmb* marker as a glioma therapeutic target.

In this study, we have performed genetic and immunohistochemical evaluation of human gliomas to determine the incidence, distribution, and pattern of localization of GPNMB antigens in brain tumors. In order to evaluate the therapeutic potential of GPNMB as an HGL-associated antigen, a series of 28 newly diagnosed HGL biopsy specimens were examined for *gpnmb* RNA transcript levels by real-time reverse transcription-polymerase chain reaction (RT-PCR). In addition, a larger panel of 77 newly diagnosed HGL cases, including the 28 samples in the mRNA study, was assessed for GPNMB protein expression by immunohistochemical analysis, and survival analyses were performed based on these two parameters. We have demonstrated that significant numbers of HGLs overexpress GPNMB at the mRNA and protein levels, and conclude that therapeutic strategies designed to target GPNMB may be feasible in the treatment of malignant glioma.

Materials and Methods

Cell Lines and Cell Transfection. Human malignant glioma (MG)-derived cell lines D54 MG, D245 MG, D247 MG, and D392 MG were established and maintained in our laboratory; T98G (MG), U251 MG, and the SK-Mel-28 (melanoma, MEL) cell lines were obtained from the ATCC. MG and MEL cell lines were grown in Zinc Option medium supplemented with 10% FCS (ZO-10% FCS); the propagation, storage, and testing of these cell lines to ensure the absence of HeLa cell contamination, inter- or intra-cell line contamination, or *Mycoplasma* infection have been published previously (22). The THRG cell line (20, 21), a genetically defined human fetal astrocyte line transfected to express GPNMB, was used as the positive control cell line for GPNMB expression.

For transient expression experiments, the ECD of *gpnmb* (nucleotides 1-1458, adenosine of the start codon as 1) was ligated into the mammalian expression vector pcDNA3.1 (Invitrogen, Carlsbad, CA). The resulting pcDNA3.1-$GPNMB_{ECD}$ was introduced into HEK293 cells by using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA), and transiently transfected cells were harvested 48 hours later. To generate stable GPNMB-transformants, the EcoRI-XhoI fragment of the pWD77 vector containing the full-length *gpnmb* cDNA (a generous gift from H. P. Bloemers, University Nijjmegen, The Netherlands) (14) was cloned into the retroviral vector pBabeBleo or pBabeNeo (23). The glioma cell lines D247 MG, D54 MG, and U251 MG were infected with retrovirus as described previously (21), and stably transfected clones were selected in medium containing 200 μg/ml of Zeocin (Invitrogen, Carlsbad, CA) or 1 mg/ml Geneticin (Gibco, Carlsbad, CA).

cDNA Cloning of *gpnmb* from Glioma Cells. For cDNA cloning of the *gpnmb* gene from human glioma cells, mRNA was isolated from D392 MG cells using the FastTrack 2.0 Kit (Invitrogen, Carlsbad, CA). After cDNA synthesis using random primers and SuperScript II RNaseH$^-$ Reverse Transcriptase (Life Technologies, Rockville, MD), the DNA fragment encoding the ECD of GPNMB was amplified by one cycle of 95°C for 2 minutes followed by 30 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute with the following primers.

$GPNMB_{ECD\ FWD}$ (sense): 5'-ATGGAATGTCTCTACTAT-3',

$GPNMB_{ECD\ REV}$ (anti-sense): 5'-GTTTGCCATCCTTAAAGG-3'.

The PCR products were further subcloned into pCR2.1TOPO vector (Invitrogen, Carlsbad, CA) and sequenced on both strands using ABI377 automatic sequencer (Applied Biosystems, Foster City, CA).

Quantitative Real-Time RT-PCR Assay. Total RNA was isolated from subconfluent cultured cells and sample tissues using the RNeasy Mini Kit (Qiagen, Valencia, CA), and then treated with RNase-free-DNase I (Ambion, Austin, TX). Total RNA sample of normal adult whole brain was purchased from Clontech (Palo Alto, CA). Total RNA (0.2 μg) was converted to cDNA with random primers and SuperScript II RNaseH$^-$ Reverse Transcriptase (Life Technologies, Rockville, MD) in a reaction volume of 20 μl. After the first-strand cDNA synthesis, 280 μl of water was added to the reaction mixture, and 2 μl of diluted cDNA sample was subjected to real-time PCR measurements.

DNA sequencing of RT-PCR products revealed an alternative splicing of *gpnmb* RNA transcripts in human glioma cells as described in Results (Fig. 1). To determine the expression

levels of each *gpnmb* RNA transcript relative to that of *β-actin*, the following primers were used in real-time PCR:

GPNMB A (sense) 5'-CACTTCCTCAATTATTCTAC-3'

GPNMB B1 (antisense) 5'-TAAAGAAGGGGTGGGTTTTG-3'

GPNMB B2 (antisense) 5'-TTGTCACCAGCAGGTCCTAA-3'

GPNMB B3 (antisense) 5'-TGGGGTGTTTGAATCATAAG-3'

β-actin$_{FWD}$ (sense) 5'-CCAACCGCGAGAAGATGACCCAGATCATGT-3'

β-actin$_{REV}$ (antisense) 5'-GTGAGGATCTTCATGAGGTAGTCAGTCAGG-3'

Primer B1, paired with primer A, amplified the overall *gpnmb* mRNA (both of the wild-type and the spliced variant), while primer B2 was located spanning the exon-exon junction to detect only the wild-type *gpnmb* transcripts (*gpnmb*$_{wt}$). Primer B3, complimentary to the insert sequence generated by alternative splicing, was specific to the splice variant form of *gpnmb* mRNA (*gpnmb*$_{sv}$) (Fig. 1B). *β-actin* transcript was amplified simultaneously as an internal control in each sample. For the PCR reactions, 2 μl of cDNA, 5 μl of 2X SYBR Green PCR Master Mix (Applied Biosystems, Warrington, U.K.), and 400 nM of each primer were used in a total volume of 10 μl. Cycling parameters were 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles (45 cycles for the detection of splice variant, *gpnmb*$_{sv}$) of 95°C for 15 seconds and 58°C for 30 seconds. Incorporation of SYBR Green dye into the PCR products was monitored with an ABI PRISM 7900HT Sequence Detector System (Applied Biosystems, Foster City, CA). Integrity of PCR products was confirmed by dissociation curve analysis (SDS 2.0 software, Applied Biosystems, Foster City, CA) and by an agarose gel electrophoresis. To normalize the degradation of total RNA used in cDNA synthesis, the threshold cycle (C_T) values were

determined for *gpnmb* and corresponding *β-actin* genes in each sample, and the *gpnmb/β-actin* ratio was calculated from the following formula (24).

$$gpnmb/\beta\text{-}actin \text{ ratio} = 2^{(C_T \beta\text{-}actin - C_T gpnmb)}$$

Relative *gpnmb* mRNA levels were expressed in terms of fold induction rate over control normal whole brain sample, which was determined by dividing *gpnmb/β-actin* ratio of tumor sample by that of normal whole brain. All measurements were performed in triplicate, and the experiments were repeated twice.

Recombinant Protein Preparation and Exoglycosidase Treatment. The ECD of GPNMB protein, $GPNMB_{ECD}$, was produced with a hexahistidine tag at the carboxy-terminus. The extracellular segment of *gpnmb* (nucleotides 64-1458) excluding the signal peptide region was cloned into a T7-based procaryotic expression vector (25). $GPNMB_{ECD}$ was expressed in *Escherichia coli* BL21-CodonPlus (DE3) RIL (Stratagene, La Jolla, CA) as inclusion bodies. After dissolution in a buffer containing guanidine, $GPNMB_{ECD}$ was purified with an Ni-NTA column (Qiagen, Valencia, CA) and renatured as described previously (26).

To produce $GPNMB_{ECD}$ protein in the baculovirus system, the same DNA fragment was fused with baculoviral vector pVL1393 (BD Pharmingen, San Diego, CA), and protein was expressed in Sf9 insect cells according to the manufacture's instructions. Log phase High Five or Sf9 cells were then infected with 100 ml of high titer viral stock and incubated while shaking at 27°C for 48 to 72 hours. After harvesting and centrifuging infected cells, the culture supernatant was dialyzed against 50 liters of 20 mM Tris pH 8.0, filtered through a 0.2-μm filter, and applied to a Q-Sepharose column. The eluate was collected by NaCl gradient, and all fractions containing protein were combined and dialyzed against 50 mM NaH_2PO_4 (pH 8.0) and

300 mM NaCl. The sample was again centrifuged; the supernatant was filtered and applied to a HisTrap Chelating HP column according to manufacturer's instructions. Eluted protein fractions were checked for appropriate molecular weight size via SDS-PAGE, and the positive fractions were combined and dialyzed against PBS, and filtered once again for sterilization.

For exoglycosidase digestion, total cell lysates of 20 μg of protein were denatured by boiling at 100°C for 10 minutes with 0.5% SDS, and then incubated with 10,000 units/ml of Peptide *N*-Glycosidase F (PNGaseF) (New England Biolab, Beverly, MA) in the presence of 1% Nonidet P 40 at 37°C for 2 hours. Samples were subjected to SDS-PAGE and immunoblotting to determine change in molecular mass.

Immunization. Immunization protocols utilized an initial DNA immunization with the plasmid vectors encoding the ECD of GPNMB, followed by boosting with the corresponding recombinant protein produced in bacteria. Rabbits were given a primary subcutaneous (s.c.) immunization with 250 μg of mammalian expression vector pcDNA3.1-*gpnmb*$_{ECD}$ or were immunized with 250 μg of bacterial recombinant GPNMB$_{ECD}$ protein emulsified 1:1 in Freund's Complete adjuvant (CFA); this was followed by 2 × boosts with 250 μg of GPNMB$_{ECD}$ protein + Freund's incomplete adjuvant (IFA) at 4-week intervals. Rabbits were bled 12 days after the last boost. Titers in serum were monitored by ELISA with GPNMB$_{ECD}$ as the capture antigen and live-cell ELISA using *gpnmb*-expressing D54 MG cells as targets.

For mice, the first immunization protocol (referred to as "B") was a combination protocol using pDNA for initial immunizations, followed by purified, bacterially expressed GPNMB protein later in the immunization protocol, since titers insufficient for fusion were obtained after pDNA immunization alone. On days 1, 49, and 77, Balb/c, C3H/He, and C57Bl/6 mice received

15 μg of GPNMB encoding pDNA in 100 μl intradermally (i.d.); 50% end-point titers vs. GPNMB protein determined with serum obtained on day 89 were less than 1/1000. The mice then received 30 μg of GPNMB protein + CFA on day 156 and a similar boost in IFA on day 176. The 50% end-point serum titers vs. source of GPNMB determined on day 188 were in excess of 1/5000. Following a minimum interval of 30 days, Balb/c recipients were boosted intraperitoneally (i.p.) with 5 μg of protein, and spleens were harvested for fusion 3 to 4 days later. From two separate fusions using protocol B, MAbs A3 and G11 were obtained. The second immunization protocol (U) consisted solely of protein immunization: day 1, 30 μg of GPNMB + CFA; days 21, 42, and 63, 15 μg protein + IFA. The 50% end-point serum titers obtained on day 74 were in excess of 1/10,000. On day 105, C57Bl/6 recipients were boosted and spleens harvested as described above; the anti-GPNMB U MAbs were derived from this fusion.

Fusion, Isolation, and Screening of GPNMB Reactive MAbs. Fusions were performed with the nonimmunoglobulin-secreting Kearney variant of P3X63/Ag8.653 by using our standard procedure (27). Supernatants were screened for positivity on the bacterially derived GPNMB protein by ELISA and on THRG cells by FMAT assay (see below). Hybridoma supernatant reactivity for plated protein was performed as previously published (27), with the exception that the secondary reagent used was goat anti-mouse IgG-Fc specific (Sigma, St. Louis, MO); the tertiary reagent was horseradish peroxidase (HRP)-Streptavidin (Zymed; South San Francisco, CA); and development was by the SigmaFast *o*-phenylenediamine dihdrochloride kit (Sigma) according to manufacturer's instructions.

Fluorometric Microvolume Assay Technology (FMAT) Analysis. Analysis of MAb binding (supernatant samples or purified MAbs) to intact cell surfaces was performed on the FMAT 8100 HTS system (Applied Biosystems, Foster City, CA). THRG cells grown in 10%FCS-ZO were harvested and fixed with 10% formaldehyde/PBS for 6 minutes at room temperature, then plated into an FMAT 96-well plate at a concentration of 10,000 cells/well; 30 µl of purified anti-GPNMB MAbs or hybridoma supernatants was added to the wells; the positive primary antibody control was the serum pool derived from immunized spleen donors, and negative background controls included 10%FCS-ZO (hybridoma supernatant control), 1% BSA/PBS buffer, or irrelevant isotype controls (IgG_1 or IgG_{2b}) at identical concentrations to the primary reagents. Secondary antibody goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) coupled to FMAT-blue dye according to manufacturer's instructions (Applied Biosystems, Foster City, CA) was added to the wells at a final concentration of 0.13 µg/ml in 1%BSA/PBS, and the plates were incubated for 2 hours in the dark at room temperature before measuring emitted fluorescence (650-685 nm) with FMAT 2.0.1 software.

Western Blotting. Total cell pellets were lysed in buffer (50 mM Tris-Cl, pH 8.0/150 mM NaCl/1% Nonidet P 40/1 mM phenylmethylsulfonyl fluoride/0.045 mg/ml aprotinin). Aliquots of sample lysates or purified GPNMB protein were subjected to electrophoresis on Tris-glycine SDS polyacrylamide gel and blotted onto polyvinylidene difluoride (PVDF) membranes according to the standard method (28). Nonspecific binding sites were blocked by using 3% nonfat milk in PBS-0.05% Tween 20. Incubations with primary antibodies were carried out overnight at 4°C with rabbit anti-GPNMB antiserum #2640 (5 µg/ml) or MAbs (10 µg/ml) in

PBS-0.05% Tween 20 containing 1% milk; irrelevant IgG_1 or IgG_{2b} was used to control for non-specific binding. After washing membranes, specific protein bands were detected using HRP–linked secondary antibodies (Amersham Biosciences, UK) and developed with SuperSignal West Pico Chemiluminescence Kit (Pierce, Rockford, IL) according to manufacturer's instructions.

Indirect Analytical Flow Cytometry (IAFC). Our standard procedures for these assays have been published extensively (22, 27, 29, 30). IAFC was performed as previously described (30) on a Becton Dickinson FACSort equipped with Lysys software (Becton Dickinson, San Jose, CA). Assays were performed at 4°C; all washes were performed with iced medium to facilitate the detection of cell surface receptors without allowing internalization to occur. As profiles obtained with cells maintained in ice cold 1% BSA-PBS or 0.5% paraformaldehyde-PBS were identical, the latter suspension buffer post secondary reagent was selected for longer-term stability. The percentage of a population designated as positive was arbitrarily defined as that region in which only the highest fluorescing 10% of the isotype control-stained cells graphed, corrected for background; this is a conservative estimate of the total positive staining population.

To examine the cell surface expression of GPNMB proteins, target cultured or biopsy-derived GBM cells were stained with anti-GPNMB MAbs G11 or U2 under nonpermeabilized conditions as described previously (30). Subconfluent cells were detached from culture flasks by incubation with 0.02% EDTA/PBS; 1×10^6 cells were maintained in 0.5% paraformaldehyde/PBS for 10 minutes at 4°C, washed, resuspended in 150 µl of PBS containing 10% FBS, and blocked for 20 minutes at 4°C. After two washes, the samples were reacted with anti-GPNMB MAbs (10 µg/ml) or irrelevant mouse IgGs (10 µg/ml) in PBS. After two

additional washes, cells were incubated with FITC-labeled secondary antibody for 30 minutes at 4°C and analyzed on a Becton Dickinson FACSort instrument (Becton Dickinson, San Jose, CA).

Quantitative FACs Determination of Receptor Density. The number of GPNMB molecules expressed per cell by cell populations was determined by quantitative FACs determination of receptor density (QFACs) using the Quantum Simply Cellular™ system (Bangs Laboratories, Fishers, IN). The microbead solution used is a mixture of five uniform populations which have varying capacities to bind no or serially increasing amounts of murine IgG; incubation of the bead sample with an identical aliquot of fluoresceinated MAb used for cell analysis allows extrapolation of antibody molecules bound/cell, and assuming 1:1 stoichiometry, of the number of receptors present, expressed as a population mean or median. The optimal concentrations of directly fluoresceinated MAbs were determined by repeated titrations of anti-GPNMB MAbs, and the minimum concentration which yielded consistent saturation of available sites on these cells was determined to be 5 μg/ml. Background binding was determined by inclusion of fluoresceinated irrelevant isotype controls (IgG_1 or IgG_{2b}) and cell surface GPNMB negative cell controls (U251 MG). Beads or cells were labeled for 2 *hours* at 4°C with 300 μl of fluoresceinated MAb (5 μg/ml), washed twice with ice-cold 1% BSA-PBS, and resuspended for analysis as described for IFAC. Analysis of receptor density was performed by interpolation with the bead standard curves using QuickCal™ software (Bangs Laboratories); positive population percent was determined as described above for indirect analysis. The consistency of labeling intensity by each batch of fluoresceinated antibody was established by calibrating the FACSort with blank and calibrating QC Windows microbead standards (Sigma Immunochemicals, St. Louis, MO), and assay-by-assay determination of microbead standard

curves (Quantum Simply Cellular Microbeads). Each assay included positive and negative cell line controls providing quality control for the directly fluoresceinated preparations, which were optimally stable at 4° C through at least 90 days. The techniques for disaggregation of biopsy and xenograft-derived cells and preparation for flow cytometry have been thoroughly described (30); briefly, cells were disaggregated in 0.4% collagenase, filtered through sterile 22-μm nylon mesh, and if necessary, further isolated from the interface of a lymphocyte separation medium (LSM; Organon Teknika, Durham, NC) gradient prior to inclusion in our standard QFACs protocol.

Direct fluoresceination of MAbs was performed by dialyzing MAbs against 115 mM sodium phosphate buffer, pH 7.4, adjusting the concentration to 1 mg/ml, and incubating with 70 μl of 1 mg/ml solution of *N*-hydroxysuccinimide ester of fluorescein isothiocyanate (Pierce, Rockford, IL) in dimethylsulfoxide. Following a 4-hour incubation at room temperature, the antibody solution was dialyzed at 4°C vs. several changes of PBS and then ultracentrifuged at 100,000 $\times g$ for 30 minutes; concentration was determined by spectrophotometer.

Affinity Constant Determination by Surface Plasmon Resonance. Purified $GPNMB_{ECD}$ protein was immobilized on the surface of biosensor chips for analysis by using the BIAcore^{TM}3000 (BIAcore, Inc., Piscataway, NJ). Coupling of antigen was achieved using *N*-ethyl-*N'*-(3-dimethylaminopropyl) carbodiimide/*N*-hydroxysuccinimide according to the manufacturer's instructions. The running buffer was 10 mM HEPES/150 mM NaCl/3.4 mM EDTA, pH 7.4. The MAb samples were passed over the biosensor chip at concentrations from 200 to 1000 nM. The association and dissociation rate constants (k_{assoc} and k_{diss}) and average

affinity were determined by using the nonlinear curve-fitting BIAevaluation software. K_A at equilibrium was calculated as $K_A = k_{assoc} / k_{diss}$.

Iodination, Immunoreactive Fraction Determination, and Scatchard Analysis. Details of these procedures have been previously published (27, 31, 32). Purified MAbs were iodinated by the Iodogen method (31) to a specific activity of 1 to 3 μCi/μg Ig. The immunoreactive fraction (IRF) was conventionally determined by Lindmo analysis (33) using increasing amounts of positive (THRG) or negative (P3X63/Ag8.653) cells as targets in an 18- to 24-hour assay at 4°C. In a single assay of MAbs A3 and G11, IRF was determined vs. GPNMB protein coupled to magnetic beads (34). Plotting the total divided by the specifically bound activity vs. the reciprocal of the antigen concentration yielded a linear plot, the intercept of which represents the inverse of the IRF. A modified Scatchard analysis was used to measure the binding affinity of iodinated MAbs beginning with serially diluted, labeled MAb starting at 10 μg/ml versus THRG MG cells, incubation at 4°C for 4 hours, and measurement of cell-bound activity as a proportion of input activity; nonlinear regression analysis to calculate B_{max} and K_A was performed with GraphPad Prism software (GraphPad Prism Software; San Diego, CA).

Tumor Samples. Samples of primary HGL tumors were obtained from 77 newly diagnosed patients at the Department of Neurosurgery, Duke University Medical Center, Durham, NC. The tumors were histologically diagnosed and graded as GBM (Grade IV, 60 cases), or AA (Grade III, 17 cases), according to WHO criteria (35). Among those 77 HGL patient samples, 25 GBM (Grade IV), and 3 AA (Grade III) cases were studied in quantitative real-time PCR analysis for GPNMB mRNA transcript expression. No patient had any history of

chemotherapy or radiotherapy before surgery. The samples were immediately snap-frozen in liquid nitrogen and stored at -80°C until analysis.

Immunohistochemistry. Immunohistochemical analysis of acetone-fixed (–70°C, 30 seconds) 5- to 8-μm frozen tissue sections of human tumor tissue was performed as described previously (22, 30). For detection of GPNMB, exposure to primary reagent (polyvalent rabbit antiserum #2640/irrelevant negative control rabbit IgG, or MAb G11/murine IgG_{2b} isotype control at 10 and 5 μg/ml) was performed for 1 hour at room temperature. Slides were washed in PBS, the appropriate dilution of biotinylated goat anti-mouse IgG or goat anti-rabbit IgG (Vector) established by previous titration was applied, and the slides were incubated for 1 hour. Slides were washed again in PBS, exposed to HRP-avidin complex (Vector, Standard ABC Kit) for 30 minutes, and following PBS washes, developed with diaminobenzidine (Metal Enhanced DAB Substrate Kit, Pierce, Rockford, IL). Slides were counterstained with hematoxylin, dehydrated, mounted, and read independently by two investigators, including a neuropathologist. Slides were scored on the basis of staining intensity (none to intense, 0–3), and staining distribution and localization (0%–25%, 1+; 26%–50%, 2+; 51%–75%, 3+; 76%–100%, 4+), with notation of parenchymal, perivascular, or nuclear staining. Positive tissue control was provided by D256 MG athymic rat xenografts.

Statistical Analysis. Relative *gpnmb* mRNA expression levels, IHC, and age were considered as possible predictors of survival. Cox proportional hazard regression analyses (SAS statistical analysis package, Cary, NC), including univariate and multivariate (categorical and continuous) survival analyses, were used to check for significance in predicting survival.

Categorized relative GPNMB mRNA expression levels ($gpnmb_{wt}$ [≤ 1.88 fold vs. > 1.88 fold], $gpnmb_{sv}$ [≤ 0.675 fold vs. > 0.675 fold] and $gpnmb_{sv+wt}$ [≤ 3.0 fold vs. > 3.0 fold]), IHC immunoreactivity (zero vs. positive), and age (≤ 45 years vs. > 45 years) were considered as possible predictors of survival. Patient survival was computed from date of pathology sample to date of death or last contact. Survival data was current as of September 23, 2004. A predictor was considered for the multivariate model if the p-value was less than or equal to 0.25 in the univariate model.

Results

Alternative Splicing of *gpnmb* RNA Transcripts in Glioma Cells. We have cloned the *gpnmb* gene from the human glioblastoma cell line D392 MG by isolation of poly-A mRNA and RT-PCR (Fig. 1). Through the cDNA sequence analysis of individual clones, in addition to the published *gpnmb* mRNA sequence (14), we found that one clone, EX1, had an in-frame insertion of a 36-bp fragment at nucleotide position 1019 in the ECD of *gpnmb* (Fig. 1A). By BLAST search (NCBI, Bethesda, MD), we determined that this 36-bp fragment insert created by alternative splicing completely matches the human genomic *gpnmb* intron DNA clone CTA-271G31 from chromosome 7 (GenBank No. NT033965). This 12 amino acid insertion variant is designated as $gpnmb_{sv}$ (splice variant), and the normal gene as $gpnmb_{wt}$ (wild type). In CTA-271G31, an additional 99 bp matching the $gpnmb_{wt}$ cDNA sequence was found directly downstream from this 36 bp-fragment. Further sequence analysis revealed that CTA-271G31 contains the human genomic *gpnmb* sequence. The 5' and 3' ends of each intronic fragment matched the consensus sequences for 3' acceptor site and for 5' donor site (36, 37), confirming that $gpnmb_{sv}$ is generated by the alternative splicing of *gpnmb* RNA transcripts.

Quantification of *gpnmb* mRNA in Glioblastoma-Derived Cell Lines and High-Grade Glioma Samples. After our initial screening analyses (13), we used real-time RT-PCR to measure the expression of the *gpnmb* gene in seven GBM cell lines, eight normal brain samples, and 28 cases of newly diagnosed high-grade glioma. First, human GBM-derived cell lines were analyzed for *gpnmb* mRNA expression. We have designed specific primer sets to identify the expression of $gpnmb_{wt}$, $gpnmb_{sv}$ as well as $gpnmb_{wt+sv}$ (Fig. 1B and details in Materials and Methods). As shown in Fig. 1C, all seven glioma cell lines examined expressed both types of *gpnmb* RNA transcripts; that is, the wild-type $gpnmb_{wt}$ and the splice variant $gpnmb_{sv}$. By quantitative real-time RT-PCR, D54 MG, D392 MG, and D247 MG cells exhibited high levels of overall *gpnmb* mRNA, while U251 MG, T98G, and D245 MG exhibited moderate to marginal levels, and U87 MG exhibited very low or no *gpnmb* mRNA expression (Fig. 2A). Therefore, we used D54 MG, D392 MG, and D247 MG cells in studies of GPNMB protein expression.

Real time RT-PCR analysis of $gpnmb_{sv}$ alone in the same cell line panel revealed that a subset of $gpnmb_{wt}$ positive tumors also express $gpnmb_{sv}$. Relative transcript levels of $gpnmb_{sv}$ RNA were 2- to 10-fold lower than those for $gpnmb_{wt}$, and no significant $gpnmb_{sv}$ or $gpnmb_{wt}$ transcripts were detected in normal brain samples (data not shown). Similar analysis of established glioma cell lines revealed that significant levels were present in three potential glioma target cell lines, D392 MG, D247 MG, and D54 MG; the latter two were chosen as reference controls on the basis of reproducible growth *in vitro* and *in vivo* as xenografts in athymic rodents.

We measured *gpnmb* mRNA levels in 28 HGL biopsy samples. We found that 19/25 (76%) GBM, and 2/3 (67%) AA were positive (at least 3-fold increase over normal whole brain

sample) for $gpnmb_{wt+sv}$ mRNA, measured inclusively (Fig. 2B and Table 1). A greater than 10-fold increase of $gpnmb_{wt+sv}$ mRNA transcripts was found in 8/25 (32%) GBM and 1/3 (33%) AA. As the $gpnmb_{wt}$ mRNA profile was virtually identical to the $gpnmb_{wt+sv}$ profile, it is probable that $gpnmb_{wt}$ is the predominant detected moiety (Fig. 2B). The splice variant $gpnmb_{sv}$ was detected in 7/25 (28%) GBM and 2/3 (67%) AA (Table 1). No significant expression for the *gpnmb* gene was detected in normal brain samples assayed simultaneously.

Isolation of anti-GPNMB MAbs. Though high levels of RNA transcripts can be predictive of protein levels, the presence of detectable protein in human tumor material must be established for targeting applications. Thus, we developed specific antibodies to detect the presence of GPNMB protein. We have immunized three different standard mouse strains (Balb/c, C57Bl/6, and C3H/He) by the intradermal route with cytomegalovirus-based plasmid DNA (pDNA) encoding the ECD of normal $GPNMB_{wt}$ or $GPNMB_{sv}$. Those animals were subsequently boosted intraperitoneally with bacterially expressed or insect cell–expressed $GPNMB_{ECD}$ protein.

Two separate fusions were performed as described in Materials and Methods. As derivation of MAbs specific for the extracellularly expressed portion of GPNMB was desired, supernatants from outgrowing hybridomas were screened for reactivity on the GPNMB-transfected cell line THRG, previously shown to express GPNMB (20, 21); the non-cell surface GPNMB expressing cell line U251 MG was used as the negative control in FMAT. From the two protocols (B: IgG_1 MAb A3 and IgG_{2b} MAb G11; U: IgG_{2b} MAb U2) three hybridomas were selected for cloning and further analysis. Representative indirect FACs histograms of purified MAb G11 are shown in Fig. 3A; this analysis establishes that MAb G11, generated

following immunization with pDNA of *GPNMB* and with bacterially produced GPNMB protein, is reactive with a cell surface epitope on the purposefully transfected THRG cell line and on D54 MG. Similar profiles were obtained with all of the components of this MAb panel on THRG, D54 MG, and SK-Mel-28 cell lines (data not shown); the non-cell surface gpnmb-expressing cell line U251 MG was negative with these MAbs detected by QFACS, but definitely positive by Western blot (Fig. 3B), suggesting that this cell line fails to insert the GPNMB protein in the cell membrane.

Detection of GPNMB Protein in Glioblastoma Cells. The integrity of the GPNMB coding sequence was first investigated by expressing the recombinant protein in insect cells. By Western blotting, purified rabbit anti-GPNMB IgG #2640 reacted with the lysates of Sf9 insect cells transiently transfected with the ECD of GPNMB (Fig. 4, left panel). $GPNMB_{ECD}$ expressed in Sf9 cells migrated with an apparent molecular weight of $M_r \sim 80$ kDa, which was significantly larger than the molecular weight of $GPNMB_{ECD}$ protein deduced from the amino acid composition (54 kDa). The ECD of human GPNMB contains 12 potential N-glycosylation sites; *N*-glycosidase treatment of Sf9 lysates significantly reduced the molecular size of $GPNMB_{ECD}$ almost identical to that produced in *Escherichia coli* (Fig. 4, left panel), indicating that the discrepancy in the molecular mass is due to glycosylation of the GPNMB protein. We further investigated the GPNMB protein expression in cultured human GBM cell lines. Detection of GPNMB proteins using antiserum #2640 was carried out in several cultured human GBM cells, including U251MG, D54MG, D247MG, and their GPNMB transfectants, either GPNMBwt or GPNMBsv as shown in Fig.4. In parental GBM cell lines, GPNMB proteins were detected by antiserum #2640 in U251MG, D54 MG and D247 MG as multiple bands between ~109 kDa and

~120 kDa (Fig. 4, middle and right panels), although the level of GPNMB protein expression in U251MG was low. The GPNMB protein expression increased significantly after transfection of U251MG, D54MG, or D247MG cells with GPNMB expressing vectors; the proteins exhibited in different degree of glycosylation as multiple bands; essentially, the GPNMB proteins migrated predominantly as two bands of apparent molecular weights M_r ~109 kDa and ~ 120 kDa.

To establish the restricted specificity of these MAbs, Western blots were performed using the glycosylated purified $GPNMB_{ECD}$ isolated from Sf9 cells as control and cell lysates prepared from the U251MG cell line as well as the GPNMB-transfected cell lines, U251MG and D247MG. As shown in Fig. 3B, MAbs G11 and A3 recognized the purified $GPNMB_{ECD}$ protein (~ 80 kDa mass as shown in arrow heads); this protein readily dimerizes, resulting in the higher mass bands detected by MAb A3. MAbs U2 and U3 exhibited similar patterns; U1 failed to react on Western blots following denaturing SDS-PAGE (data not shown). All MAbs detected bands between approximately 109 and 120 kDa in the GPNMB-transfected D247MG line in a pattern similar to that of rabbit polyclonal antiserum #2640 (Figs. 3B and 4). Only MAb G11 was capable of consistently identifying these bands in the untransfected glioma cell lines, U251MG and D54MG, as well as their GPNMB-transfectants in a pattern similar to that of rabbit polyclonal antiserum #2640 (Fig. 3B). Both IgG_1 (data not shown) and IgG_{2b} irrelevant isotype controls were negative. Detection of cell surface GPNMB was also performed with anti-GPNMB MAb G11. Indirect FACs analysis revealed reactivity of G11 with GPNMB-expressing D247MG cells under nonpermeabilized conditions (Fig. 3C). Furthermore, stable transfectant D247 MG-GPNMB showed a peak shift compared to that of the parental line D247 MG, indicating an increase in the number of surface GPNMB molecules after transfection. Irrelevant control IgG_{2b} was unreactive with both lines.

Quantitative Analyses of Anti-GPNMB MAbs. On the basis of cell surface antigen detection and performance in Western blot, MAbs A3, G11, and U2 were chosen for further analysis. MAbs G11 and U2 were fluoresceinated for QFACs analysis, and cell surface GPNMB densities were determined on a panel of cell lines, disaggregated xenografts, and biopsy samples (Table 2). MAb U2 was determined to be the optimal FITC-labeled reagent for these analyses. An example of such an analysis of patient biopsy GBM 2180 is provided in Fig. 5; from the regression equation calculated for FITC-MAb binding to quantitated receptor site beads, the fluorescent channel value obtained with GBM 2180 cells predicts a median GPNMB density of 7 $\times$ 10^4 GPNMB molecules/cell. Results of QFACs analyses are presented in Table 2. Fifty percent (4/8) of long-term cultured HGL cell lines expressed GPNMB in excess of 1 $\times$ 10^4 molecules/cell; only 3/9 established xenografts did so. Notable is the fact that D54 MG and D247 MG xenografts do not express GPNMB, whereas the cell lines derived from these GBM samples did. Analysis of freshly disaggregated cells from AA (n = 6) and GBM (n = 27) biopsies revealed that 1/6 (17%) of AA and 11/27 (41%) of GBM expressed cell surface GPNMB with a range of densities from 1.1 to 7.8 $\times$ 10^4; one GBM expressed > 10^5 GPNMB molecules per cell.

Positive GPNMB-expressing cell lines THRG and SK-Mel-28 exhibited 1.4 to 3.9 $\times$ 10^5 and 3 to 9 $\times$ 10^4 GPNMB molecules per cell, respectively. No GPNMB expression was detected by QFACs in medulloblastoma cultured cells, xenografts, or biopsies.

Kinetics of MAb-to-GPNMB Binding. A kinetic analysis of the interaction of purified MAbs with immobilized $GPNMB_{ECD}$ by surface plasmon resonance (BIAcore) was conducted to

determine the association and dissociation rate constants and calculation of the affinity constants. Determination of the association and dissociation rates from the sensorgrams reveals a k_{assoc} of 1.1×10^6 (1/Ms) and a k_{diss} of 1.1×10^{-3} (1/s) for MAb G11. The K_A at binding equilibrium, calculated as $K_A = k_{assoc}/k_{diss}$, is 9.6×10^8 (1/M). Anti-GPNMB MAbs A3, G11, and U2 were also each analyzed by conventional Scatchard analysis vs. the THRG cell line. The range of results for each MAb is presented in Table 3. The binding of MAb A3 was apparently compromised following iodination; the detected immunoreactive fraction was only 12%, and amounts bound for the Scatchard analysis were negligible. The probability of targeted residues in the binding site is supported by the observation that fluoresceination of MAb A3 also resulted in an inactive preparation, despite an acceptably homogeneous HPLC profile. MAbs G11 and U2 exhibited K_A values in the range of 1.7 to 4.7×10^8 M^{-1} measured by Scatchard analysis vs. cell surface expressed GPNMB and IRFs in the range of 75% to 91% for G11 and 71% to 75% for U2, respectively. The estimated cell surface receptor densities obtained from the βmax values for G11 and U2 were quite similar (ranges of $4–8 \times 10^4$) in multiple assays.

Immunohistochemical Analysis. The immunohistochemical analysis of HGL tissue samples from newly diagnosed cases was performed in two stages. In stage 1, rabbit anti-GPNMB serum #2460 was used to stain frozen sections of the tumor samples (3 AA, 24 GBM) used for GPNMB mRNA analysis. One GBM case was nonevaluable due to high background staining. As shown in Fig. 6, Mann-Whitney analysis (one sided, assuming a direct relationship between mRNA presence and production of protein) revealed that the correlation of *gpnmb* mRNA and protein expression was significant ($p < 0.033$ for AA and GBM; $p < 0.016$ for GBM alone); the higher the detected level of mRNA, the more probable a positive

immunohistochemistry result. In addition to these cases, an additional 14 AA and 36 GBM were stained with rabbit anti-GPNMB serum #2460; of these cases, 13 HGL were concurrently stained with MAb G11 and appropriate irrelevant IgG_{2b} isotype control. Results of this analysis are illustrated in Fig. 7 and summarized in Table 4 (17 cases of AA and 60 cases of GBM). As shown in Fig. 7, GPNMB localization can present in different patterns; while the majority of cases exhibit focal or multifocal tumor parenchymal staining panels (E, G, J, and K) with clear membrane staining (panels G and H), a subset of GBMs (41%, Table 4) exhibits pronounced perivascular accumulation of GPNMB either with (26%), or without (15%), accompanying parenchymal staining. Notable is the observation that none of 17 AA cases exhibited perivascular accumulation, suggesting that this may be a characteristic of the more malignant grade IV tumors.

Survival Analysis. Analyses were also conducted to examine the effect on survival of select predictors in specific patient subgroups: RNA expression data from 28 newly diagnosed patients (25 GBM and 3 AA; Fig. 8) and the IHC information form 77 newly diagnosed patients (60 GBM and 17 AA; data not shown). When these parameters were considered as predictors of survival, age was a strong predictor of survival in all analyses (data not shown). The estimated survival probability of patients with an AA or GBM who survive 1 year was 0.88 (95% CI: 0.77-1.0) for patients <=45 years of age and 0.61 (95% CI: 0.48-0.77) for patients >45 years of age. The hazard of death for older patients was 2.75 (95% CI: 1.42-5.33) times that of younger patients. For GBM patients alone the 1-year survival estimate was 0.85 and 0.58 for patients <= 45 years and > 45 years respectively (Cox hazard ratio of 2.56 [95% CI: 1.18–5.56]).

The relative $GPNMB_{wt}$ mRNA expression level was a strong predictor of survival across all analyses. The estimated 1 year survival probability was 0.86 (95% CI: 0.63 – 1.0) for HGL (AA or GBM) patients with relative $GPNMB_{wt}$ mRNA expression levels less than 1.88 fold and 0.62 (95% CI: 0.44 – 0.87) for patients with relative $GPNMB_{wt}$ mRNA expression level greater than 1.88 fold. The hazard of death for patients with relative $GPNMB_{wt}$ mRNA expression level greater than 1.88 fold was 5.10 (95% CI: 1.43 – 18.20). For GBM patients alone as shown in Fig. 8A, the 1-year survival estimate was 0.83 and 0.58 for the low $GPNMB_{wt}$ mRNA and moderate/high $GPNMB_{wt}$ mRNA groups, respectively (Cox hazard ratio of 6.76 [95% CI: 1.50–30.54]). The relative $GPNMB_{sv}$ mRNA expression level was also a strong predictor of survival. The estimated 1 year survival probability was 0.86 (95% CI: 0.63 – 1.0) for patients with relative $GPNMB_{sv}$ mRNA expression levels less than 0.675 fold and 0.62 (95% CI: 0.44 – 0.87) for patients with relative $GPNMB_{sv}$ mRNA expression level greater than 0.675 fold. The hazard of death for patients with relative $GPNMB_{sv}$ mRNA expression level greater than 0.675 fold is 3.16 (95% CI: 1.05 – 9.52). For GBM patients alone as shown in Fig. 8B, the survival probability at 52 weeks for low levels of $GPNMB_{sv}$ mRNA was 0.83; whereas, the probability for moderate/high levels of $GPNMB_{sv}$ mRNA, 0.58 (Cox hazard ratio of 6.88 [95% CI: 1.53–30.99]). Finally the relative $GPNMB_{wt+sv}$ mRNA expression level was a significant predictor of survival. The estimated 1 year survival probability was 0.71 (95% CI: 0.45 – 1.0) for patients with relative $GPNMB_{wt+sv}$ mRNA expression levels less than 3.0 fold and 0.67 (95% CI: 0.49 – 0.90) for patients with relative $GPNMB_{wt+sv}$ mRNA expression levels greater than 3.0 fold. The hazard of death for patients with relative $GPNMB_{wt+sv}$ mRNA expression levels greater than 3.0 fold was 4.93 (95% CI: 1.38 – 17.65). For GBM patients alone as shown in Fig. 8C, the survival probability at 52 weeks for low levels of $GPNMB_{wt+sv}$ mRNA was 0.67; whereas, the

probability for moderate/high levels of $GPNMB_{wt+sv}$ mRNA, 0.63 (Cox hazard ratio of 6.45 [95% CI: 1.42–29.30]). There were no statistically significant relationships between IHC immunoreactivity and survival (data not shown).

Within the context of a Cox model, a likelihood ratio test was used to compare the univariate model involving only age to the multivariate model with age and each mRNA variable. This test was conducted to assess whether the RNA variables added prognostic value beyond that provided by age. Among patients with AA or GBM $GPNMB_{wt}$ mRNA and $GPNMB_{wt+sv}$ mRNA added significant prognostic value beyond that provided by age alone ($p = 0.19$ and $p = 0.026$, respectively). The effect of $GPNMB_{sv}$ mRNA trended towards significance after adjustment for age ($p = 0.081$). Among GBM patients, all three RNA variables provided significant prognostic value beyond that provided by age alone ($p=0.014$ for $GPNMB_{wt+sv}$ mRNA, $p = 0.009$ for $GPNMB_{wt}$ mRNA, $p = 0.008$ for $GPNMB_{sv}$ mRNA).

Discussion

Effective MAb-based targeted therapy depends on several factors, which include characteristics of the target antigens, the immune reagents used, the route of administration and the tissue dynamics of the tumor (8). Preferential expression on or around tumor cells and membrane localization are major requirements for tumor antigens in immune-based therapy (3, 8).

This paper presents data which indicate that the transmembrane glycoprotein GPNMB is overexpressed in a significant portion of high-grade glioma tumors, at both the mRNA and the protein levels, but not in normal brain tissues. We have performed genetic and immuno-histochemical evaluation of human gliomas to determine incidence, distribution, and pattern of localization of GPNMB antigens in brain tumors. We used quantitative RT-PCR to analyze 28

newly diagnosed human HGL biopsy samples (3 AA and 25 GBM) received directly from surgery to determine levels of mRNA for $GPNMB_{wt}$ + $GPNMB_{sv}$. The results revealed that 76% of GBM patient samples were positive for *gpnmb* transcripts and that 32% of GBM showed a greater than 10-fold increase over normal brain in *gpnmb* mRNA expression. In contrast, only marginal levels of *gpnmb* mRNA were noted in whole brain RNA extract. Correlation between the assays was good, as Mann-Whitney analysis (one sided, assuming a direct relationship between mRNA presence and production of protein) revealed that the correlation of *gpnmb* mRNA and protein expression as detected by immunohistochemistry was significant; that is, the higher the detected level of mRNA, the more probable a positive immunohistochemistry result (Fig. 6). However, the sensitivity of mRNA detection is far higher than that of immunohistochemical assays, which accounts for some of the discrepancies noted (Fig. 6). In cases where protein is detected in tissue in the absence of mRNA, the most likely explanation is that the mRNA is short-lived and chronically produced, leaving a relatively long-lived protein *in situ* (38). GPNMB antigens exhibit diffuse staining in frozen sections with distinct membrane staining and absence of blood vessel staining by both polyvalent rabbit antiserum and MAbs demonstrably specific for GPNMB. These observed staining patterns corroborate the cell membrane staining observed in QFACs assays as described above. These data demonstrate the establishment of specific and reliable antibody probes for analysis of tissue and biopsy-derived cell populations.

Concerning subcellular localization, indirect FACS analysis with anti-GPNMB MAb G11 under nonpermeabilizing conditions demonstrated that GPNMB proteins are expressed at the surface cell membrane of human glioblastoma cell line D247 MG. Furthermore, a shift of peak was observed following stable transfection with full-length GPNMB, indicating an increase in

the number of surface GPNMB protein molecules following transfection. This supertransfectant cell line will likely prove useful in our preclinical MAb localization and tumor immunotherapy models.

Although some extracellular matrix antigens, such as tenascin, and some non-internalizing surface antigens are suitable for immunotherapeutic approaches, the ideal antigens for brain tumor immunotherapy would be those that fulfill the following criteria: (A) the general consensus for minimum surface antigen density is $\geq 1 \times 10^3$ protein molecules per cell (30, 39), (B) stability at the cell surface and lack of antigen shedding (8), and (C) internalization following binding to ligand or antibody (8). Glioma cell surface-expressed GPNMB fulfills these criteria in terms of adequate cell surface diversity, dynamics of protein synthesis, half-life, internalization, and mechanisms of degradation.

Little is known about the distribution and function of GPNMB proteins in normal human organs (13, 14, 40). However, for tumors localized within the CNS, the optimal route for the administration of MAb-based therapeutic agents is through surgically created resection cavities or saturation of an entire hemisphere by intracranial microdiffusion (41). The expression of GPNMB in distant normal tissues should not compromise the compartmental delivery of GPNMB-related immunological agents within the CNS, because only small amounts of conjugates reach systemic organs (42).

The mechanistic biological significance of the aberrant expression of GPNMB in high-grade gliomas remains to be determined. In this study, the expression levels of *gpnmb* RNA transcripts showed a positive correlation with increasing grade of tumors. The GBM group exhibited higher *gpnmb* mRNA levels than those in AA; in addition, the appearance of perivascular accumulation of GPNMB protein was noted in GBM, but was absent in AA. The

ECD of GPNMB contains 12 potential N-glycosylation sites, an RGD integrin-binding motif, and a heparin-binding motif (14). Other functional motifs found are a polycystic kidney motif and a proline-rich region that presumably forms a hinge and can mediate protein-protein interaction. In a genetically defined human glioma model using minimally transformed human fetal astrocytes (20), transfection of GPNMB resulted in the drastic change of tumor phenotype, with invasion of brain tissues and formation of spontaneous metastasis (21). Thus, GPNMB may serve as an adhesion molecule mediating cell-cell and/or cell-matrix interaction (15) and may contribute to the acquisition of the invasive nature of malignant glioma cells.

For survival analysis, newly diagnosed AA/GBM patients over the age of 45 had a higher risk of death, as has been known for decades. Similarly, in this population (AA/GBM or GBM alone) univariate analyses show that patients with moderate/high relative GPNMB mRNA expression levels (wt, sv, and wt+sv) had a higher risk of death. The relative GPNMB mRNA expression levels (wt, sv, and wt+sv) held the same significance, even after controlling for age of patient. Taken together, the results of survival analysis suggest that the relative GPNMB mRNA levels represent a strong prognostic predictor of poor GBM/AA patient survival. There are only a few molecular markers that are prognostic for survival in malignant gliomas. High GPNMB expression in HGL patient samples, strong GST-π protein expression in human gliomas (43), and a functional polymorphism in EGF gene (44) are associated with clinically more aggressive gliomas and are useful and powerful prognostic marker of poor patient survival.

In conclusion, increased *gpnmb* mRNA levels correlated with elevated GPNMB protein expression in HGL biopsy samples and higher risk of death. Statistically significant predictors of survival among patients with GBM only or GBM/AA are age and the mRNA expression variables (wt, sv, wt+sv). In a Cox model examining the joint effect of age and RNA in these

populations mRNA expression was shown to add significant prognostic value beyond that provided by age alone. These data indicate that GPNMB is a potentially useful tumor-associated antigen in immunotherapeutic approaches for malignant gliomas. We propose that therapeutic strategies designed to target GPNMB may be successful in malignant glioma treatment.

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Table 1. Incidence of *gpnmb* mRNA expression in high-grade gliomas from newly diagnosed patients*

Diagnosis	AA	GBM
Overall *GPNMBwt+sv* transcripts		
Positive [a]	2/3 (67%)	19/25 (76%)
> 10-fold [b]	1/3 (33%)	8/25 (32%)
Splice variant *GPNMBsv*		
Positive [a]	2/3 (67%)	7/25 (28%)

*Abbreviations used: AA, anaplastic astrocytomas; GBM, glioblastoma multiforme.

[a] Detected by real-time PCR analysis; positive cases were defined as those with an *gpnmb* RNA levels 3-fold higher than normal brain.

[b] More than 10-fold greater than normal whole brain sample.

Table 2. Quantitative FACs analysis for GPNMB expression by HGL and MED cell lines, xenografts, and biopsy-derived cell populations

	Cultured Cells		Xenografts		Biopsies	
	positive cases / total cases	Est. gpnmb density*	positive cases / total cases	Est. gpnmb density*	positive cases / total cases	Est. gpnmb density*
HGL	4/8[a] (50%)	1.1–7.4 × 10^4	3/9[b] (33%)	1–5 × 10^5	12/33 (36%)	1.1–7.8 × 10^4[c]
Medulloblastoma	0/4	0	0/3	0	0/1	0

* Molecules of cell surface GPNMB as calculated by QFACs with MAbs (G11, U2) assuming 1:1 stoicheometry.

[a] The Positive cell lines included D54MG, D245MG, D392MG, and T98G; U251 MG and D247 MG are negative by QFACs, but definitely positive by Western blot (Fig. 3B); notable negative cell lines: U87 MG.

[b] D54 MG and D245 MG xenografts were negative for cell surface GPNMB, while the analogous cultured cell lines were positive.

[c] One GBM biopsy sample expressed an estimated 3 × 10^5 GPNMB molecules/cell.

Table 3. Determination of affinity and IRF of anti-GPNMB MAbs and the density of GPNMB on cells

				Affinity constant $K_A(M^{-1})$		
MAb	No. of Assays	IgG Class	IRF[a]	$GPNMB_{ECD}$	THRG cells	Estimated # of GPNMB per cell
A3	1	IgG_1	12%	NE[b]	NE	
G11	3	IgG_{2b}	75–91%	9.6×10^8	$1.7\text{-}3.1 \times 10^8$	$3.5\text{-}8.6 \times 10^4$
U2	2	IgG_{2b}	71–75%	2.7×10^7	$2.1\text{-}4.7 \times 10^8$	$5.2\text{-}8.2 \times 10^4$

[a]IRF was determined for A3 and G11 in one assay on GPNMBecd-coated beads; all subsequent IRF assays were performed on pelleted THRG cells.

[b]MAbs A3 and G11 were assayed in the same experiment; the IRF value for A3, concurrent with a > 90% value for G11, suggested that iodination had affected the antigen binding site, as HPLC profiles before and after labeling were identical; subsequent determination of input bound in Scatchard analysis corroborated the assumption of iodination damage.

Table 4. Summary of immunohistochemical evaluation of frozen AA and GBM tissue sections from newly diagnosed patients with polyvalent anti-GPNMB antiserum #2640 and/or MAb G11

Cases	AA	GBM
N	17	60
Positive*	12/17 (71%)	39/60 (65%)
Positive tumor parenchymal staining	12/12 (100%)	23/39 (59%)
Prominent perivascular staining	0/12 (0%)	6/39 (15%)
Both parenchymal and perivascular staining	0/12 (0%)	10/39 (26%)

*13 cases (AA and GBM) were stained concurrently with both antiserum #2640 and MAb G11; in 12/13 cases (92%) the result (+ or -) and staining pattern observed were completely concordant. There was a slight tendency for staining with MAb G11 to be more intense (Fig. 7, panels B and G). The single discrepancy was a GBM sample positive with MAb G11, exhibiting a diffuse parenchymal pattern; it was negative with antiserum #2640.

Figure Legends

Figure 1. Schematic protein domain structure of human GPNMB and its alternatively spliced insertion in glioblastoma-derived cell lines. (A) NH_2, amino terminus; SS, signal sequence; RGD, RGD tripeptide; PKD, polycystic kidney disease domain; TM, transmembrane segment; COOH, carboxyl terminus; amino acid residue numbers lie above and below the structure. **Alt. splice**, 12-amino acid insert generated by alternative splicing as described in this paper. DNA sequence and predicated amino acid translation of the 36-bp insert segment are indicated. (B) Alternative splicing of *gpnmb* RNA transcripts generating a 36 bp-insert (*gray box*) in the ECD. Positions of oligonucleotide primers used in RT-PCR analyses are indicated by arrows. (C) RT-PCR performed with primer pairs in (B). Amplification of overall *gpnmb* transcripts ($gpnmb_{wt+sv}$), the wild-type ($gpnmb_{wt}$), and the splice variant ($gpnmb_{sv}$) *gpnmb* mRNA. *Bottom*; *β-actin* amplification. *Lane M*; 100 bp-DNA ladder.

Figure 2. Comparison of *gpnmb* mRNA expression levels in glioblastoma cell lines and quantification of *gpnmb* mRNA in high-grade glioma samples. (A) Overall $gpnmb_{wt+sv}$ mRNA levels were measured in seven human GBM cell lines by quantitative real-time RT-PCR using primer pair A and B1. Relative *gpnmb* mRNA levels are expressed in terms of fold induction rate over normal whole brain sample. Results are mean ± SD of triplicates. (B) Relative expression levels of *gpnmb* mRNA. Results are the mean of triplicate real-time PCR measurements repeated twice in two pathological groups: AA, anaplastic astrocytoma (n = 3); GBM, glioblastoma multiforme (n = 25); and NWB, normal whole brain. Filled rectangle: $gpnmb_{wt+sv}$; Open rectangle: $gpnmb_{wt}$; hatched rectangle: $gpnmb_{sv}$.

Figure 3. Detection of GPNMB on glioma and GPNMB-positive cells. (A) Indirect FACs analysis. Reactivity of MAb G11 for formalin-fixed, nonpermeabilized D54 MG and THRG cells (dotted lines). Filled peaks represent control staining with normal mouse IgG_{2b} for THRG and D54 MG. (B) Western blot analysis of GPNMB protein by anti-GPNMB MAbs. Composite immunoblot analysis of MAbs G11, A3, and irrelevant isotype control IgG_{2b} against $GPNMB_{ECD}$ protein purified from insect cells Sf9 (as shown on the right-end side of each gel panel) and whole cell lysates (20 μg) prepared from U251MG, D247MG and/or their GPNMB-transfectants. Irrelevant isotype control IgG_1 was also negative (not shown). The locations of protein molecular weight markers are shown to the left side of the panels. The apparent molecular weights for glycosylated $GPNMB_{ECD}$ purified from Sf9 insect cells are indicated by arrow heads (~80 kD) and the GPNMB proteins detected in human glioma cell lines and transfectants represent differentially glycosylated forms as shown in brackets between ~109 and ~120 kD. (C) Reactivity of G11 for formalin-fixed, non-permeabilized D247 MG cells (solid line) and D247 MG cells transfected with full-length GPNMB (dotted line). The filled peak represents control staining with normal mouse IgG_{2b} for D247 MG. Note the increase in MAb binding following transfection (shift to the right) indicating a higher number of cell surface GPNMB molecules than in the parental cells.

Figure 4. Western blot analysis of GPNMB protein by rabbit antiserum # 2640. Total cell lysates containing 20 μg of protein were separated by SDS-PAGE and probed with anti-GPNMB rabbit antisera #2640. *Left panel:* Cell lysates from insect Sf9 $GPNMB_{ECD}$ transfectants were incubated with or without *N*-glycosidase (PNGase F), and electrophoresed along with recombinant $GPNMB_{ECD}$ protein produced in bacteria *E. coli*. Note the mobility shift after *N*-

glycosidase treatment from M_r ~ 80 kDa to ~54 kDa. *Middle & right panels:* Detection of GPNMB proteins in cultured human GBM cells, including U251MG, D54MG, D247MG, and their GPNMB transfectants, either $GPNMB_{wt}$ or $GPNMB_{sv}$ as shown on top of the gel. Note the GPNMB protein expression increased after transfection; the protein migrated predominantly as two bands of apparent molecular weights M_r ~109 kDa and ~ 120 kDa. The locations of protein molecular weight markers are shown to the side of the panels.

Figure 5. Quantitative FACs (QFACs) analysis of biopsy GBM 2180-derived cells with anti-GPNMB MAb U2. Nonpermeabilized cells, noninternalizing conditions. Estimated median cell surface density: 7×10^4 GPNMB molecules per cell.

Figure 6. Correlation of GPNMB mRNA and protein expression; Mann-Whitney one-sided test. Prot+ and Prot- : positive and negative tissue samples as detected by immunohistochemistry. $p < 0.033$ and $p < 0.016$ for AA plus GBM and GBM alone, respectively; one-tailed test; medians significantly different.

Figure 7. Immunohistochemistry. Frozen sections of GBM tissues were reacted with normal rabbit IgG (panels A, D, and J), purified IgG from rabbit anti-GPNMB antiserum 2640 (panels B, E, H, and K), murine IgG_{2b} (panel G), or anti-GPNMB MAb G11 (panels C, F, and I) at a concentration of 10 μg/ml. Irrelevant staining controls not illustrated (murine IgG_{2b} as control for panels C and F, normal rabbit IgG as control for panel H) were identical to the control illustrated. Note the absence of staining in panels A, D, G, and J. Panels A, B, C: GBM 1917; 50X. The staining pattern of GPNMB in this GBM is primarily perivascular, with weaker

cytoplasmic staining in the surrounding tumor parenchyma. Panels D and F: GBM TB 1943; 100X. The predominant staining pattern in this GBM is focal, with distinct membrane decoration. Panel E: GBM TB 1399; 132X. Similar to the pattern displayed by TB 1943, staining in this GBM is also focal and membrane-associated (➔). Panels G-I: GBM TB 1944; 100X. This GBM exhibits focal cytoplasmic staining of tumor cells invading normal brain. Panels J and K: GBM TB 526; 132X. The staining pattern in this specimen is diffuse cytoplasmic (moderate) with some distinct membrane decoration and perivascular accumulation. Note that staining patterns obtained with both the polyvalent anti-GPNMB antiserum 2640 and MAb G11 are identical in pattern for a given specimen.

Figure 8. Kaplan-Meier curve of time to progression stratified by (A) $gpnmb_{wt}$ RNA transcript levels, (B) $gpnmb_{sv}$ RNA transcript levels, and (C) $gpnmb_{wt+sv}$ RNA transcript levels for newly diagnosed GBM patients.